THREE-DIMENSIONAL QUALITATIVE AND QUANTITATIVE ANALYSIS OF MOUSE CORNEAL NERVE

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Introduction

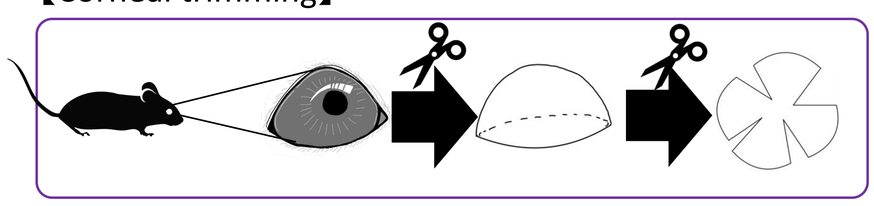
Corneal sensory nerves (CSNs) have attracted considerable interest as **a potential site for the assessment of diabetic peripheral neuropathy**. In rodent models of diabetes, CSNs are an important marker for peripheral neuropathy. However, the previous analyses were limited to a two-dimensional method, and now there is an increasing need for more accurate three-dimensional evaluations. Therefore, **we establish a method to elucidate the three-dimensional structure of the mouse corneal nerve and quantitative analysis.**

Materials and Methods

Male C57BL/6 mice aged 8 weeks were used. Corneal nerve fibres were visualized using a transparency technique and immunofluorescence. Wholemount images were acquired to construct three-dimensional images using confocal microscopy and analyzed with Imaris software. The density and total length of the nerve fibres running in all directions were then calculated.

Sample collecting → **Staining** → **Tissue clearing** → **Image-acquisition** → **Image-processing** → **Statistical analysis**

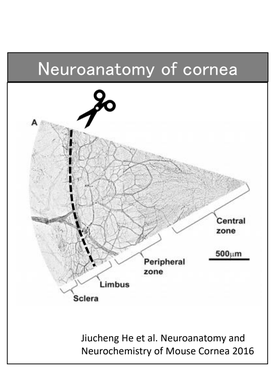
【Corneal trimming】

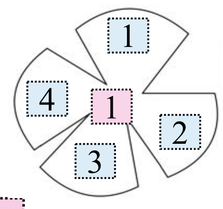


【Staining and tissue clearing】

	Fixing	Washing	Degreasing		Blocking	Immunostaining			Transparency	Watching
Solution	4%PFA	TBS	Scale CUBIC-1	TBS	Goat serum	β 3Tubulin	TBS	AlexaFluor 488	Scale CUBIC-2	Mounting
Temperature	4℃	4℃	37℃	4℃	37℃	37℃	4℃	37℃	37℃	4℃
Time	1 day	1 day	3 days	2-3 times	1 day	3 days	2-3 times	3days	1 day	

【Analysis area】



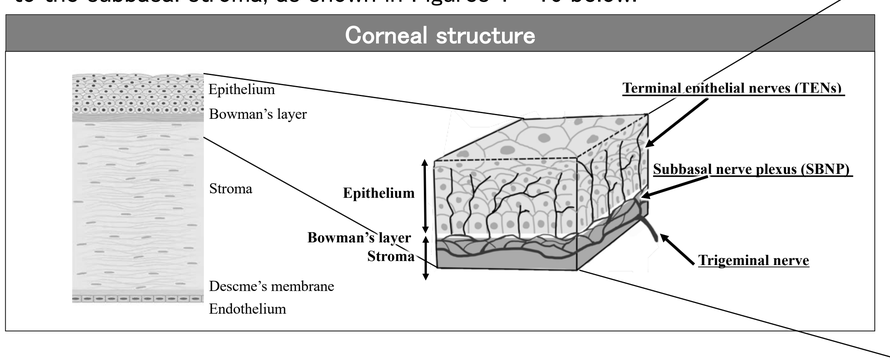


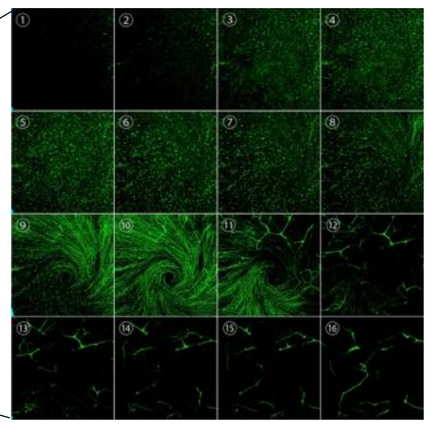
1 Central zone

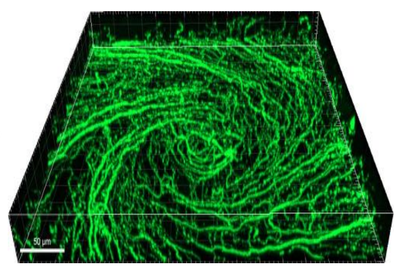
1 ~ 4 Peripheral zone

200～500 μm from the edge of the cornea

Full thickness Z stacks were obtained from the superficial corneal epithelium to the subbasal stroma, as shown in Figures 1～16 below.



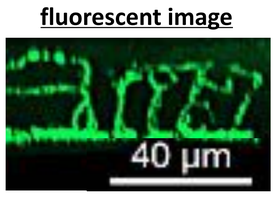




【Image-processing/analysis】

Two modules of analysis were utilized in a semi-automated manner to segment SBNP and TENs from image background. **The Surfaces tool** was used to remove background and manually set absolute intensity threshold. Then, faux-3D image was reconstructed comprising three Z-dimension slices. **The Filament Tracer tool** was selected for use with the 'Threshold' algorithm enabled, feature pre-processing disabled. Neurite filaments were then traced semi-automatically to fill small gaps between discontinuous fibers. Total volume (μm^3) for SBNP, total volume/density and length (μm) for TENs was then measured from the resulting reconstruction using the statistics tool.

fluorescent image



Surface tool

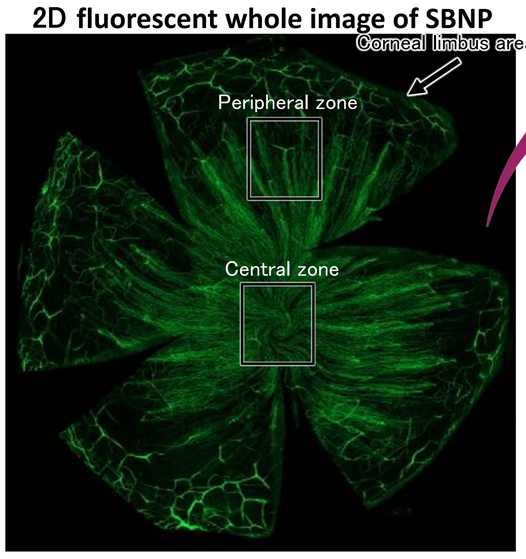
→ for the volume/density

Filament tool

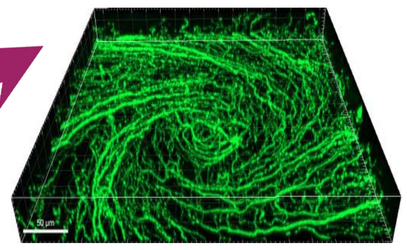
→ for the length

Results

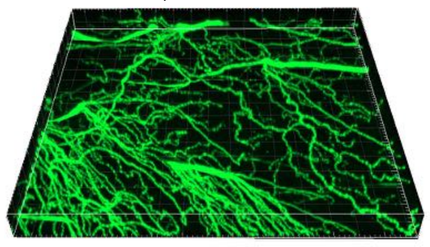
2D fluorescent whole image of SBNP



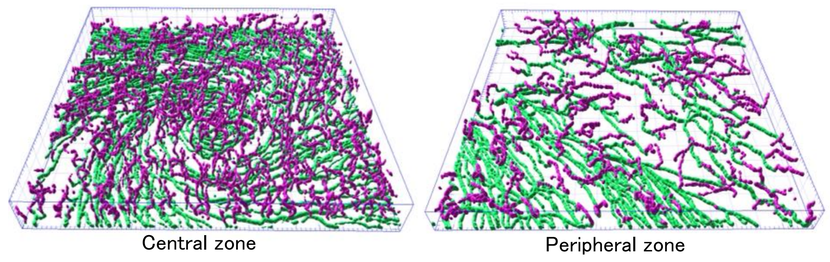
Central zone



Peripheral zone



The reconstructed 3D image (by Surface tool)



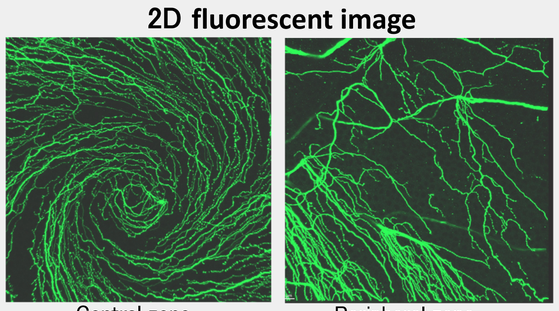
Central zone Peripheral zone

SBNPs spiral run in the basal layer of the cornea (green) and TENs extend from the SBNPs through the basal layer into the corneal epithelium (red). Each nerve fibre is isolated and later analysed quantitatively using parameters..

Subbasal nerve plexus(SBNP)

The subbasal nerve plexus (SBNP) branched from the bundles of nerve fibres in the corneal limbus stroma and formed whorl-like structures or vortices in the subbasal zone towards the centre of cornea.

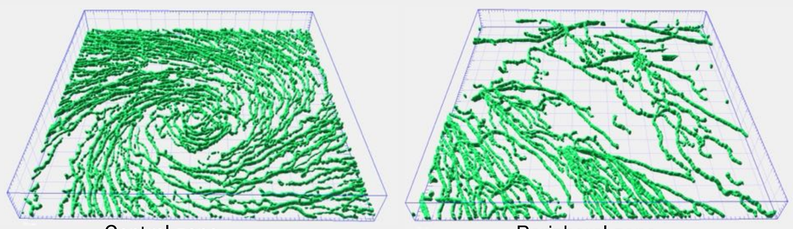
2D fluorescent image



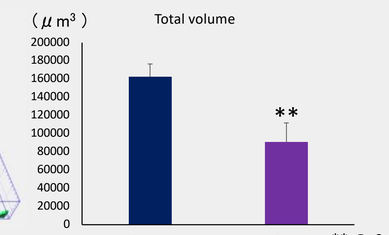
Central zone Peripheral zone

3D analysis for the nerve volume (by Surface tool)

The total volume of SBNP was significantly larger in the centre than in the periphery.



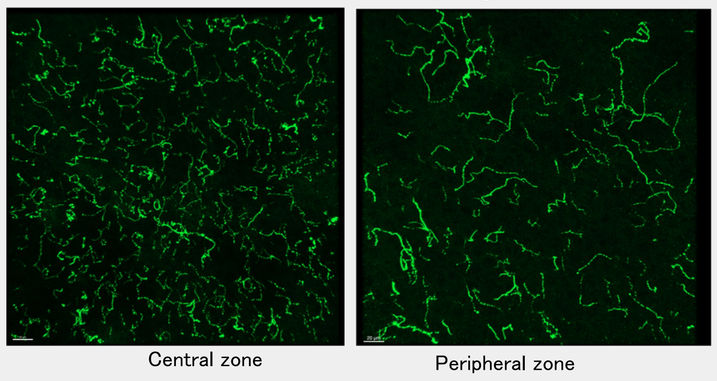
Central zone Peripheral zone



Terminal epithelial nerves(TENs)

The TENs branched from the SBNP and extended vertically to the epithelial surface in the central cornea, whereas in the peripheral cornea, they extended vertically to the epithelial surface and then ran parallel to it

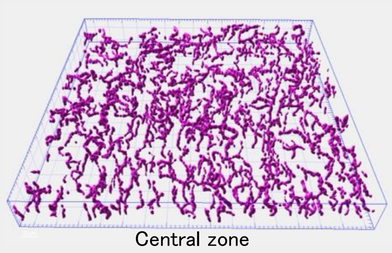
2D fluorescent image



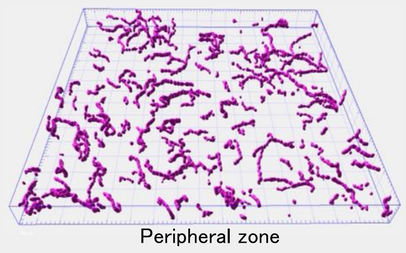
Central zone Peripheral zone

3D analysis for the nerve volume (by Surface tool)

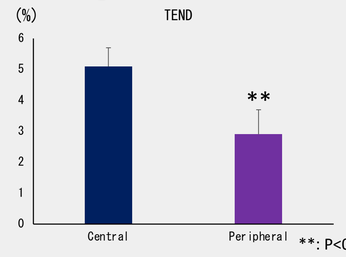
The density of TENs (TEND) was significantly higher and total volume was also larger in the centre.

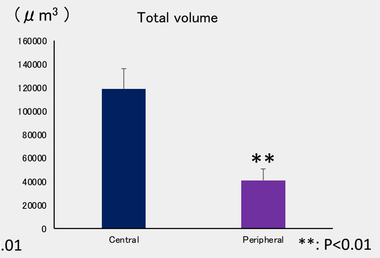


Central zone



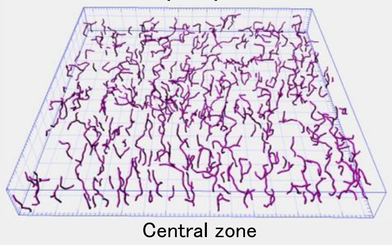
Peripheral zone



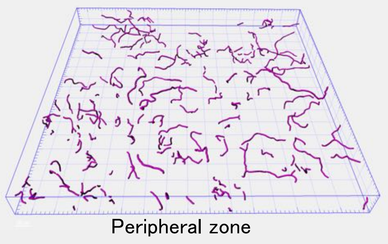


3D analysis for the nerve length (by Filamment tool)

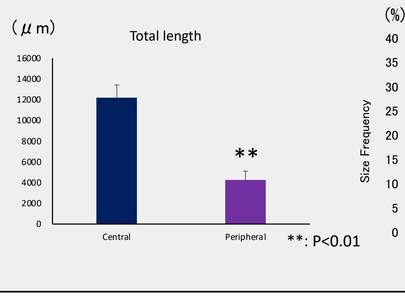
The total length of nerve fibers was longer compared to the periphery. The histogram also show a higher number of longer nerve fibres at the peripheral cornea.

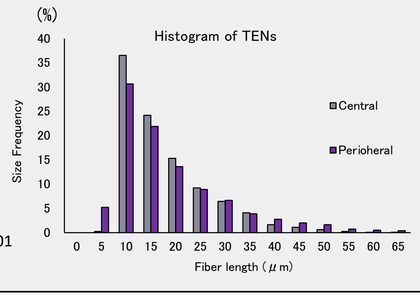


Central zone



Peripheral zone





Conclusion

This study successfully captured the microstructure of mouse corneal nerve fibres and quantified the resulting three-dimensional images.